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Hyperthermia as an Adjuvant to Chemotherapy and Cytokine  
Immunotherapy for the Treatment of Breast Cancer

PRINCIPAL INVESTIGATOR: Michele Pritchard

CONTRACTING ORGANIZATION: Health Research, Incorporated  
Buffalo, New York 14263

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## Body

Year number two was challenging, and therefore was of great training value. Both the training and research accomplishments are outlined below with special attention paid to unexpected difficulties in the experimental procedures.

This predoctoral grant was written to examine the effect of combining fever-range whole body hyperthermia (WBH) with Doxil chemotherapy (task I) and IL-12 cytokine immunotherapy (task II) in animal models of breast cancer. Progress in year number 2 has recently improved with the implementation of a new animal model that closely represents human breast cancer and will replace the not as appropriate CT26 model (a colon cancer model) used in year number one. This addition represents the most significant advance of year number two. Secondly, the work with the CT26 model has moved forward slightly and the importance of IFN- $\gamma$  to the observed enhancement of the anti-tumor response with the combination of IL-12 and whole body hyperthermia noted in year one has been hypothesized. Unfortunately, a bizarre, septic shock-like syndrome has been observed in three separate experiments and has hampered further progress in both animal models the past 4 months. The etiology of this syndrome is still under investigation by the PI and the Roswell Park Cancer Institute Department of Laboratory Animal veterinary staff.

***Implementation of the 4T1 animal model:*** In February of 2001, Suzanne Ostrand-Rosenberg, Ph.D. from the University of Maryland came and gave a talk at Roswell Park Cancer Institute that described her use of the 4T1 cell line as an animal model to study breast cancer. The PI had the opportunity to meet with Dr. Ostrand-Rosenberg and at that time, established a collaboration with her. The 4T1 cell line is a murine mammary adenocarcinoma syngenic to balb/c mice that, when implanted into the mammary fat pad, *spontaneously metastasizes* to the draining lymph node, lung and liver in the mice. Secondly, the tumors later metastasize to the central nervous system. The model recapitulates the human situation to a large degree and is widely accepted as a good animal model. Another advantage of this model is the way in which tumor metastasis can be quantified in each organ by exploiting the fact that the 4T1 tumor cells are 6-thioguanine resistant. By simply harvesting organs of interest, digesting them using various enzymes and later plating out the resultant single cell suspension in medium containing 6-thioguanine, the number of tumor cell clones can be enumerated by counting colonies on the bottom of a tissue culture dish. Because it is usually the metastases that kill cancer patients, we believe that this is a relevant model to use to evaluate our treatment protocols.

Although only preliminary experiments have been performed suggesting that IL-12 decreases lung metastasis (see Figure 1, pg 8 in the Appendix), the model is now solidly in place in the laboratory. Experiments can now be done which test the efficacy of both Doxil/doxorubicin and IL-12 used in combination with WBH, not only on the primary tumor but also on metastases to satisfy the proposed work in both Tasks I and II.

***Mechanism of action for the enhanced anti-tumor effect of IL-12 and WBH in mice bearing CT26 tumors:*** Work has also continued with the CT26 model that has helped elucidate more clues to the mechanism behind the enhanced anti-tumor effect of IL-12 used in combination with WBH. Many of the biological effects of IL-12 occur due to its ability to induce the production of a potent inflammatory cytokine, IFN- $\gamma$ . Not only does IL-12 play a large role in creating a Th1 environment due to this induction of IFN- $\gamma$  but it also initiates the non-immune mediated, anti-tumor effects via the induction of anti-angiogenic factors namely, IP-10 and Mig. The last successful experiment that was carried out looked at the production of IFN- $\gamma$  in the serum of mice treated with IL-12 and WBH. This was only a preliminary experiment as only 3 mice per group were used; however, there is a distinct trend in a higher amount of IFN- $\gamma$  in the serum of mice treated with the combination compared to IL-12 alone at the 48 hour time point post WBH (see Figure 2, page 9 in the Appendix). Control animals or animals treated with WBH alone did not produce detectable levels of IFN- $\gamma$ . This experiment has been repeated and serum samples from 5 mice per group at 24 and 48 hours post WBH have been sent for cytokine analysis. Immunohistochemistry and western blotting is currently being performed to look for differences in the amount of IP-10 and Mig in the tumors of these same animals.

***Unexpected difficulties with the experimental protocol:*** The paucity of reportable data was due to the injection-related illness in the experimental animals at various points post WBH. In three separate experiments (two CT26 and one 4T1), many if not all animals experienced what could only be described as a shock-like syndrome. It is characterized by swollen, red tails, whitish eyes, labored breathing, hunched appearance, wobbly-ness and lethargy in the mice within 5 minutes of the i.p. IL-12 or control buffer injection. It occurred after the first injection of the second cycle of IL-12 treatment in two experiments and after the first injection of the first cycle in one experiment. The first two incidents happened within a week of one another. As this phenomenon occurred in animals receiving control buffer as well as those receiving IL-12, one of the veterinarians at Roswell Park Cancer Institute, Elizabeth Hansen, DVM hypothesized that it was due to LPS contamination of the PBS used to make the buffer. She suggested that all future buffers be made with sterile, non-pyrogenic saline for injection. This was done for the following experiment, and the catastrophe occurred after the first injection. Necropsies on animals the PI sacrificed to prevent further suffering were performed by another veterinarian, Thomas Martin, DVM. The gross examination revealed a trauma, circulatory in nature noted to the greatest degree in the lungs. Histopathologic analysis is still underway. In addition, tests on these same buffer/cytokine aliquots are to be performed in the near future with the assistance of either Dr. Hansen or Dr. Martin. As the buffers are confirmed sterile, we are currently hypothesizing that there is something in the BSA used as a carrier protein for the IL-12 (and present also in the control buffer) that cannot be removed by sterile filtration with a 0.22  $\mu$ m filter apparatus. This has been a quite disappointing, tragic and unexpected difficulty. Further experiments cannot be performed until the etiologic agent is identified.

***Training experiences of year two:***

- A. The development of the collaboration with Dr. Ostrand-Rosenberg was a valuable learning experience. Gaining enough confidence to approach an established scientist outside of the PI's Institute and generate such collaboration will undoubtedly assist in future endeavors of the same caliber. In addition to the acquisition of the 4T1 cell line, the PI has gained tremendous assistance on how to use the model and suggestions for experiments from Dr. Ostrand-Rosenberg as well.
- B. The setback hypothesized to be associated with the buffers used in these experiments was judged by the PI as a tragic one. However, learning how to troubleshoot and work with others to solve the problem has been enlightening and of great educational value.
- C. Completed work on IL-12 and WBH was presented this past April at the combined annual meetings of the North American Hyperthermia Association and Radiation Research Society in San Juan, Puerto Rico. The abstract submitted for a poster presentation (a reproduction of the abstract is included in the Appendix, pg. 10) was also chosen for a short slide presentation. The training value of this experience was invaluable, not only due to the experience of putting together and discussing a poster, but also in preparing and delivering a brief, yet informative slide presentation to an audience of professionals in the field.

### **Key Research Accomplishments**

- Implementation of a new and highly relevant animal model with which the PI can continue the experiments outlined in the Statement of Work
- Elucidation of IFN- $\gamma$  as a potential mediator of the enhancement of the anti-tumor immune response when utilizing a combination treatment of IL-12 + WBH
- Participation in the combined Annual Meetings of the North American Hyperthermia Society and Radiation Research Society was a noteworthy highlight of the completed work.

### **Reportable Outcomes**

No manuscripts were written

One abstract (see Appendix, pg 10) was presented at the combined Annual Meetings of the North American Hyperthermia Society and Radiation Research Society in San Juan, Puerto Rico, in April 2001.

Two presentations were given:

1. A one hour Departmental seminar on the work outlined in the above abstract

2. A six minute talk at the combined Annual Meetings of the North American Hyperthermia Society and Radiation Research Society in San Juan, Puerto Rico, in April 2001.

### **Reportable Outcomes, continued**

No patents or licenses were applied for and/or issued.

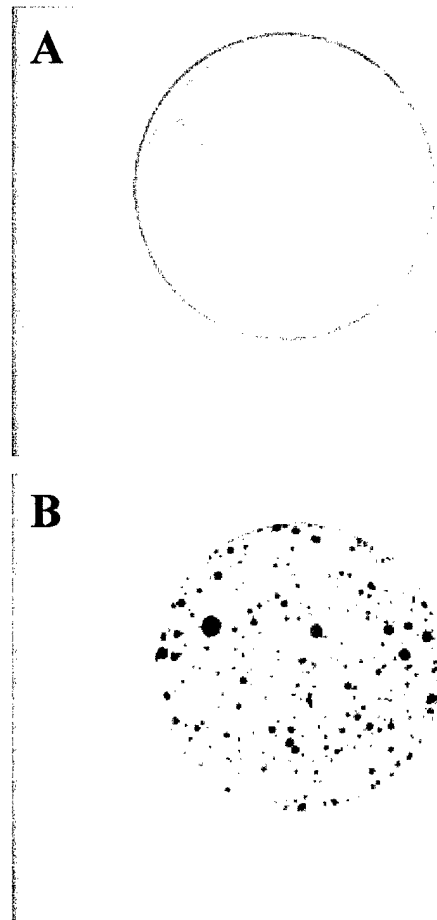
No degrees were conferred.

No development of cell lines, tissue of serum repositories; informatics occurred.

No funding applied for.

No employment or research opportunities have been applied for and/or received based upon the experience/training supported by this award.

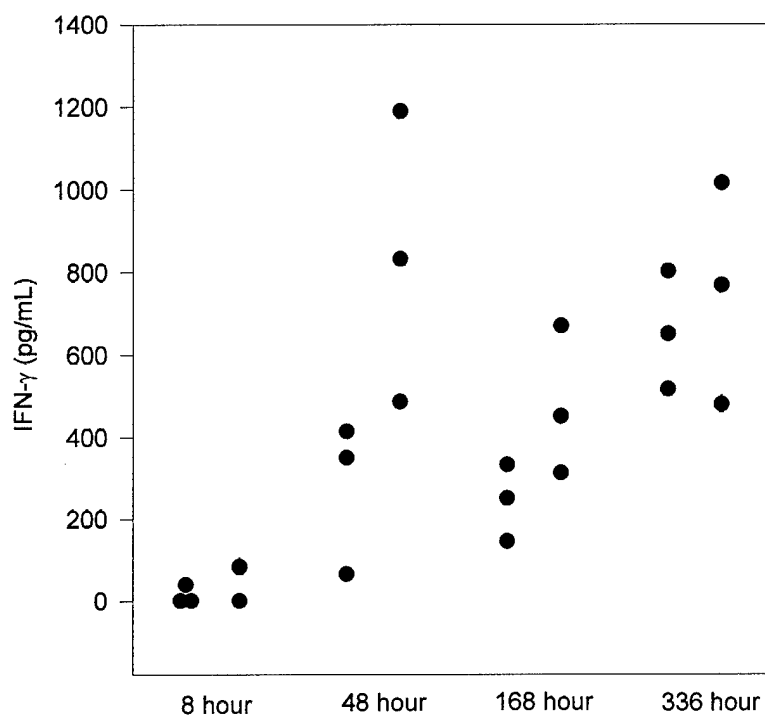
## Appendix



**Figure 1. IL-12 treatment of 4T1-bearing mice appears to decrease the number of tumor metastasis to the lung.** Mice bearing established (4x5mm) tumors were treated with IL-12 for 5 days. On day 15 the animals were sacrificed, lungs harvested and digested in collagenase IV and elastase at 4<sup>0</sup> C for 75 minutes. This digested material was filtered through a 70  $\mu$ m filter and the resultant single cell suspension was diluted 1:10 and plated out in medium containing 6-thioguanine. Ten days after plating, the tumor cell colonies were fixed in methanol, rinsed with distilled H<sub>2</sub>O and then stained with methylene blue. A. tumor from the lung of a control animal. At the 1:10 dilution, the number of tumor cells was so great that distinct colony borders were unidentifiable. Greater dilutions will need to be done in future experiments. B. Tumor colonies from the lung of an IL-12 treated animal. Here, a 1:10 dilution was adequate for enumeration of colonies. These plates are representative of three animals per group.



## Appendix continued



**Figure 2. Enhanced serum IFN- $\gamma$  concentrations are present in mice treated with IL-12 used in combination with WBH at 48 hours post WBH.** Mice were treated with WBH such that their core body temperatures were maintained at  $39.8 \pm 0.2^\circ\text{C}$  for 6 hours. Controls were subject to all the manipulations as were the heated animals, yet they remained at room temperature. Immediately following WBH, the first dose of IL-12 was administered. Treatment continued for five days, off for two and then the cycle was repeated. At 8, 48, 168 and 336 hours post WBH, blood was drawn from each mouse, the serum from which was subjected to murine IFN- $\gamma$  analysis by ELISA (R&D Systems). Blue circles indicate animals treated with IL-12 alone and red circles indicate those animals that received the combination treatment. We could not detect IFN- $\gamma$  in the serum of control animals or those that received WBH alone (DNS). (n=3 per group)

## **Appendix continued**

This abstract was presented at the combined Annual Meetings of the North American Hyperthermia Society and the Radiation Research Society, San Juan, Puerto Rico April 2001. It was accepted for a poster and a short slide presentation

**Effects of fever range, whole body hyperthermia on the anti-tumor efficacy of interleukin 12 treatment in tumor bearing, Balb/c mice.** Michele T. Pritchard, Stanley F. Wolf<sup>§</sup> and Elizabeth A. Repasky, Roswell Park Cancer Institute, Buffalo, NY 14263, USA <sup>§</sup>Genetics Institute, Cambridge, MA 02140, USA.

It is well established that administration of interleukin 12 (IL-12) results in potent anti-tumor activity in murine models. Fever range, whole body hyperthermia (FR-WBH) has been shown to have several immunomodulatory effects. Used alone, a modest yet reproducible anti-tumor effect can be observed. In this work, the combination of FR-WBH with a low dose (100ng/day; i.p. for 14 days) of free IL-12 was tested in Balb/c mice bearing CT26 tumors to determine whether an enhancement of the effects of IL-12 could be obtained. Our results indicate that combining 100ng of IL-12 with FR-WBH is as effective as 300ng IL-12 alone, yet produces fewer side effects. We have also found that FR-WBH can enhance the survival of mice receiving 100ng of IL-12 per day when compared to appropriate controls. The greater efficacy of the combination therapy seems to correlate with higher levels of IFN- $\gamma$  in the serum of those animals early during the course of the IL-12 treatment. Timing of FR-WBH with respect to that of the initiation of IL-12 treatment is important. An inhibition or lack of enhancement of the anti-tumor effect of IL-12 is observed if the first IL-12 dose is given prior to FR-WBH or 12 hours after the FR-WBH treatment, respectively. Finally, mice cured of their disease for at least 5 months by the combination treatment were able to resist a normally tumorigenic dose of CT26 cells suggesting the development of a memory response. These data suggest that a novel immunomodulatory treatment regimen including IL-12 and FR-WBH may have significant clinical benefit compared to either modality alone.